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(54) Title: AN ACTIVATED-THIOL POLYMER FOR DRUG DELIVERY

(57) Abstract: The synthesis and characterization of an acrylamide copolymer for use as a carrier for the delivery of water soluble drugs. The polymer contains active-sulfhydryl groups for coupling of ligands through a disulfide linkage. The polymer can also be prepared containing pendant amino groups in addition to the active-sulfhydryl moiety. This allows the use of different chemistries to conjugate a variety of ligands to the polymer.

TITLE

AN ACTIVATED-THIOL POLYMER FOR DRUG DELIVERY

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The present invention was made under a NIH grant D.E.R, 5R29AI34278. The United States Government may have right to this invention.

FIELD OF THE INVENTION

This invention relates to an activated acrylamide copolymer with activated thiol groups, and conjugates of the copolymer.

BACKGROUND OF THE INVENTION

Antisense oligonucleotides have shown great potential as therapeutic agents in several areas including anticancer and antiviral chemotherapy. One limitation to their successful development as therapeutics is their poor uptake by mammalian cells. Many approaches to increase uptake efficiency have been examined, but none have achieved wide scale acceptance. Development of a better delivery system is still being explored worldwide to take antisense to clinical usage.

Water soluble macromolecular carrier systems have shown great potential in drug delivery due to their diversity in physicochemical and biological properties which can improve the chemical stability and pharmacokinetics of the conjugated drug. One promising macromolecular carrier is the synthetic N-(2-hydroxypropyl)methacrylamide (HPMA) polymer. The polymer alone exhibits little or no binding to cell surfaces in a number of test systems (rat visceral yolk sacs and hepatocytes), exhibits little immunogenicity, and is captured by cells at a rate consistent with fluid-phase pinocytosis. The pinocytotic uptake of soluble HPMA copolymers by cultured cells was dependent on the Mw of polymers. Usually the higher the Mw the lower the rate of cell capture. But some cultured cells, such as rat intestinal sacs, showed an increased rate of pinocytic capture with increasing polymer Mw, which is proposed to be due to adsorptive-pinocytosis.

The Mw of HPMA copolymers also influences in vivo behavior, such as, the rate of excretion and pharmacological activity. The blood-clearance of HPMA homopolymer preparations (Mw = 28 -91 kD) has been studied following intravenous administration to rabbits. It was shown that polymers of higher Mw had a slower rate of clearance from the blood stream, and the limiting molecular-weight threshold for glomerular filtration was determined to be 45 kD. Therefore, the size alone of the HPMA carrier can provide a variable element to optimize the drug delivery system for a specific therapeutic purpose.

N-(2-hydroxypropyl)methacrylamide polymer (HPMA) has shown great potential as a carrier for targeted delivery and controlled release of small molecule pharmaceutics. Successful targeted delivery has been achieved using a variety of targeting moieties including, carbohydrate residues (galactosamine and fucosylamine), proteins (hormones or transferrin), and antibodies. For controlled release the drug molecules have been conjugated to HPMA polymer through peptide bonds using a reactive p-nitrophenyl ester co-monomer. Since p-nitrophenyl ester is relatively unstable in aqueous solutions, this reaction usually needs to be performed in organic solvents in order to obtain high yields. In aqueous solution, hydrolysis of the active ester is expected to compete for the desired aminolysis, especially when the amino groups are carried by large molecules, such as peptides, antisense oligonucleotides and proteins. Therefore, coupling efficiency in aqueous solution is expected to be low for these molecules. Additionally, coupling through a peptide linkage requires proteolytic degradation for release of the drug. This requires a lysosomal route of uptake which can place biopharmaceutics such as peptides and nucleic acids in the presence of degradative enzymes.

Objects of the Invention

It is, therefore, an object of the invention to provide an acrylamide copolymer that can be conjugated with water-soluble molecules through a disulfide bond in an aqueous environment.

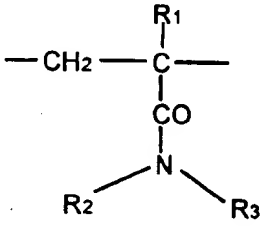
Another object of the invention to provide a copolymer that can be conjugated to form pharmacuetic carriers.

Further objects of the invention will become evident in the description below.

BRIEF SUMMARY OF THE INVENTION

The present invention involves a copolymer of substituted acrylamide-type monomers and an active thiol-containing monomer, a pyridyldithio-acrylamide. This composition enables coupling in an aqueous environment, and does not require a nonaqueous solvent environment to carry out conjugation with disulfide-bond containing compounds.

An embodiment of the present invention is a copolymer comprising, substituted acrylamide monomeric units which are the same or different and of the formula;



where

R_1 is H, methyl or ethyl,

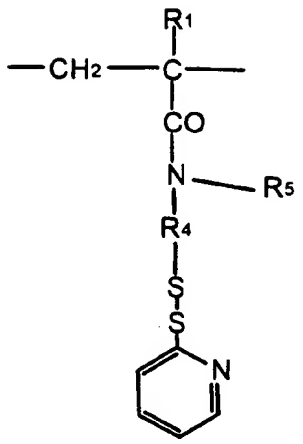
R_2 and R3 are the same or different and are

alkyl having one to eight carbon atoms or alkoxyalkyl having one to twelve carbon atoms and one to three -OH groups, or

hydroxyalkoxyalkyl of two to twelve carbon atoms and one to three hydroxy groups, or

aminoalkyl having two to twelve carbon atoms and one to three amino groups, and

pyridyldithio-acrylamide monomeric units which are the same and different and of the formula;



where R_4 is

an alkyl chain having one to eight carbon atoms or alkoxyalkyl having one to twelve carbon atoms and zero to three -OH groups, or

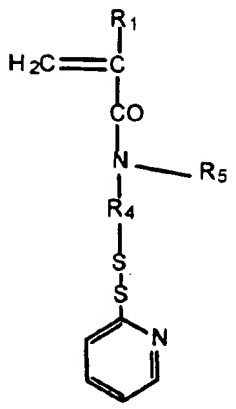
an hydroxyalkoxyalkyl chain of two to twelve carbon atoms and zero to three hydroxy groups, and

R_5 is H, methyl, or ethyl,

the mole percent of the substituted acrylamide monomeric units in the copolymer being between 70 and 99 the remaining monomeric units being pyridyldithio-acrylamide monomeric units.

In a preferred embodiment, R_3 is H, methyl, or ethyl and R_2 is the alkyl or alkoxyalkyl, as defined, R_4 is the alkyl or alkoxyalky, and R_5 is H. Preferably, the acrylamide monomeric units are derived from N-(2-hydroxypropyl) methacrylamide, i.e. R_1 is methyl, R_2 is hydroxypropyl, and R_3 is H. For the pyridyldithio-acrylamide monomeric units R_1 is preferably methyl, R_4 is ethyl, and R5 is hydrogen. In another embodiment of the invention, all or at least a portion of the acrylamide monomeric units are amino-alkyl, preferably aminopropyl.

Another embodiment of the invention is a momomeric composition of the formula;



where R_1, R_4, and R_5, are defined as above.

The nitrogen atom in the aromatic ring in the above compositions may be at the 2 position as shown, or in the 4 position, opposite the bond to the sulfur atoms.

Another embodiment of the invention is a conjugate of the copolymer defined above and an agent bonded through a disulfide bond. The copolymer of the present invention can be conjugated through the disulfide bond with any agent containing a free sulfhydryl group. These include, for example, small molecule pharmaceutics, peptides, proteins, and oligonucleotides containing sulfhydryl groups.

Another embodiment is a process for forming a conjugate which comprises providing the copolymer defined above and contacting the copolymer in an aqueous environment with an agent with a free sulfhydryl group to form a conjugate of the copolymer and the agent through a disulfide bond.

The present invention involves an active thiol-containing acrylamide co-polymer. Coupling to the polymer of water soluble molecules, such as an antisense oligodeoxynucleotide, occurs rapidly and in high yield. Since the drug and polymer are conjugated through a disulfide bond, the conjugate is expected to be stable in the bloodstream but cleaved intracellularly by thiols such as glutathione and/or redox enzymes. It has been found that an antisense-oligonucleotide/polymer conjugate is efficiently internalized by cultured cells.

As an example of the invention, an activated-thiol monomer, N-[2-(2-pyridyldithio)] ethyl methacrylamide (PDTEMA; Figure 1), and incorporated it into N-(2-hydroxypropyl) methacrylamide (HPMA) co-polymer (Figure 1). The

PDTEMA/HPMA co-polymer serves as a carrier for delivery of pharmaceutic agents, especially antisense therapeutics. Similarly, PDTEMA/HPMA/APMA (N-(3-aminopropyl) methacrylamide) co-polymers can be used.

PDTEMA/HPMA and PDTEMA/HPMA/APMA co-polymers have been found to be stable and have a long shelf-life. As such, the copolymers of the invention can be stored in their active form until needed; at which time the desired pharmaceutic agent can be readily coupled. In addition to the pharmaceutic agent, other desired funtionality's can be attached to enhance the performance of the drug carrier. The other functionality's can include, but are not limited to, targeting moieties for cell or organ specific delivery, fusogenic agents that allow for lysosomal escape of the drug, and transport agents that allow for a non-endocytotic mode of uptake.

A variety of chemistries can be used for coupling of the pharmaceutic agent and other functionality's, and the same or different chemistries can be used for each. The primary mode of coupling is via the activated pyridyldithio-acrylamide monomer (e.g. PDTEMA). Any agent possessing a free sulfhydryl group will react with the activated monomer in aqueous solution resulting in conjugation of the agent to the polymer via a disulfide bond. The coupling of the pharmaceutic via a disulfide bond is an important advantage of the copolymers of the invention. This type of linkage is expected to be stable in the blood stream but readily cleaved within the cell, allowing intracellular release of the drug.

Other chemistries can be used to generate non-cleavable conjugates. These types of couplings may be especially advantageous for the non-pharmaceutic functionality's (i.e., targeting moieties, etc.). For instance, the active sulfhydryl can be reduced on the pyridyldithio-acrylamide monomer to generate a free sulfhydryl group. Subsequently, maleimido or iodoacetal derivatized agents can be reacted with the free sulfhydryl to produce non-cleavable thiol ether linkages. In addition, for copolymers with alkylamino chains, in substituted acrylamide monomeric units, e.g. PDTEMA/HPMA/APMA co-polymer, agents can be coupled to the pendant amino group on the chain using amine specific reagents such as succinimidyl or imido-esters.

Advantages over Prior-art Systems

The copolymers of the present invention offer several advantages for use as pharmaceutic carriers.

- Simple and inexpensive to synthesize
- High stability and long shelf-life
- Great flexibility in coupling chemistries
- Provides for stable serum transport of the drug, yet allows efficient intracellular release

Practical Applications:

The polymer is designed for use as a carrier for delivery of pharmaceutics, especially water soluble molecules such as antisense therapeutics. It can be used for both targeted and non-targeted delivery. Its utility and effectiveness can be enhanced with the use of agents that can allow lysosomal escape or complete bypass of an endocytotic mechanism of cellular uptake.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of a reaction scheme for synthesis of N-[2-(2-pyridyldithio)ethyl methacrylamide (PDTEMA) and its incorporation into HPMA co-polymer.

Figure 2 is a FPLC chromatogram of co-polymer A eluted from a gel permeation column. UV absorbence was measured at 280 nm (solid line). The refractive index is indicated by the dotted line. Column: Pharmacia HR10/30, Buffer: pH 7.3 PBS, Flow rate: 0.4 mL/min, Chart speed: 0.5 cm/min.

Figure 3 is a UV spectrum of co-polymer A in 0.1 M pH 7.0 phosphate buffer and 2-thiopyridone in 0.1 M buffer B at pH 9, 10 or 11, as indicated.

Figure 4 is a graph illustrating the kinetics of the hydrolysis of copolymer A at pH from 8 to 13 as monitored by the absorbence at 320 or 341 nm.

Figure 5 is a schematic showing the chemical structure of sulfhydryl pAntp peptide (pAntp-SH) and fluorescein labeled oligonucleotide (RSS-OSH). The underlined portion of RSS-OSH indicates the sequence of the oligonucleotide which contains phosphorothioate linkages.

Figure 6 is a graph illustrating the kinetics of the conjugation reaction between pAntp-SH and copolymer A at pH 3 to 8 as monitored by the absorbence at 341 nm.

Figure 7 is a photograph of samples examined by gel electrophoresis showing kinetics of the conjugation of oligonucleotide to polymer C at different pHs as indicated. Aliquots of the reaction mixtures were removed and run into denaturing 20% polyacrylamide gels immediately at the times indicated. For the pH 7.0 and 8.0 reactions, 20 hour's samples are also shown. The control reactions were treated with 0.1 mM DTT for 30 minutes.

Figure 8 is a UV spectrum of oligonucleotide-HPMA conjugate (dotted line), oligonucleotide-peptide-HPMA conjugate (solid line), and HPMA co-polymer (dashed line). The HPMA co-polymer sample was prepared from the same pyridyl disulfide modified HPMA copolymer but was treated with 1.0 mM L-cysteine to release the active pyridyl groups. The polymer was purified away from the released thiopyridone by G-50 chromatography.

Figure 9 is a photomicrograph showing cell uptake as determined by confocal microscopy. HeLa S3 cells were pre-cultured in DMEM medium containing 10% fetal bovine serum on 1.5 cm diameter cover slips for 2-3 days, such that about 10% of the cover slip was covered by cells. The media was replaced and the free oligonucleotide OSH (left panel) or the oligonucleotide-HPMA conjugate (right panel) was added to a final oligonucleotide concentration of 5 μM. After 5 hour incubation in a CO2 incubator, the cells were rinsed with PBS three times and fixed using acetone. The coverslips were fixed to slides using nail polish and observed through a 100X oil immersion objective on a Nikon Diaphot 200 confocal microscope equipped with an Omnichrome argon laser 150. Pictures were taken with a digital camera attached to the microscope and integrated 5 times.

DETAILED DESCRIPTION OF THE INVENTION

Examples

Materials and Methods

Chemicals and instruments

N-(2-Hydroxypropyl) methacrylamide (HPMA), N-(3-aminopropyl) methacrylamide (APMA) hydrochloride, 2',2'-azo-bis(isobutyronitrile) (AIBN) and methacryloyl chloride were purchased from Polyscience Inc. 2-Aldrithiol (DPDS) was from Aldrich, cysteamine and Dulbecco's modified eagle's medium (DMEM) nutrient mixture were purchased from Sigma. HeLa S3 cells (Human epithelial carcinoma cells) were from American Type Culture Collection. Other chemicals or reagents were from Aldrich or Sigma. NMR-spectra were recorded on a Bruker AC200 NMR spectrometer. Mass-spectra were obtained on a Thermabeam Mass detector. UV spectroscopy was performed using a Varian Cary 3E spectrophotometer. Buffer A (McIlvaine's citric acid-phosphate buffer pH 3.0 through 8.0) was prepared by combining 0.5 M citric acid and 0.5 M disodium phosphate in a suitable ratio to give the desired pH. Buffer B (Sorensen's glycine II buffer pH 9.0 through 13.0) was prepared by combining suitable volumes of 0.5 M glycine in 0.5 M NaCl with 0.5 M NaOH.

Sulfhydryl peptide (Figure 5, pAntp-SH) and disulfide modified oligonucleotide (Figure 5, RSS-OSH) were synthesized by the University of Utah DNA/Peptide core facility. Peptide was purified by HPLC and stored at -70° C in the elution buffer containing approximately 1% TFA. The oligodeoxynucleotide phosphorothioate was synthesized on an Applied Biosystems 394 automated DNA synthesizer on a 1.0 μmole scale using standard phosphoramidite chemistry, except that 3H-1,2-benzodithiol-3-one 1,1-dioxide replaced aqueous iodine in the oxidation step. For the oligodeoxynucleotide synthesis, most reagents were from Applied Biosystems Inc. except Spacer 9, thiol-modifier C6 SS and fluorescein phosphoramidite were from Glen Research. The oligonucleotides were purified using 20% PAGE and recovered by electroelution followed by ethanol precipitation.

Synthesis of N-[2-(2-pyridyldithio)] ethyl methacrylamide (PDTEMA)

5.135 g DPDS (23 mmoles) was dissolved in 60.0 mL 95 % EtOH in a 250 mL round bottom flask. A solution of 1.223 g cysteamine (16 mmoles) in 60.0 mL de-ionized water (pH approximately 8.5) was added drop-wise over 10 minutes to the DPDS solution with stirring at room temperature. After incubation for 1.5 hour at room temperature, the solvent was removed by rotary evaporation at 35° C. The product was obtained as a syrup which was dried by co-evaporation 3 times with 6 mL of anhydrous pyridine.

The dried crude product was dissolved in a mixture of 25.0 mL anhydrous DMF and 50 mL anhydrous pyridine. N_2 was bubbled through for 5 minutes to remove oxygen. A magnetic stirrer was added and the bottle was sealed tightly with a rubber stopper. 3.10 mL methacryloyl chloride (32 mmoles) was added slowly over 5 minutes using a syringe. After stirring the solution for 2 hours at room temperature, the reaction was quenched by adding 400 mL saturated $NaHCO_3$ and extracted twice with 400 mL ethyl acetate. The combined organic phase was washed with 2 x 100 mL of de-ionized water and dried using anhydrous $MgSO_4$. The clear solution was evaporated to dry at 35° C by rotary evaporation. The crude product was dissolved in $CHCl_3$ and absorbed to about 10 mL silica gel, which was then applied to a short silica gel column (12.5 x 5.5 cm, packed with hexane). The column was eluted with a hexane:EtOAc concentration gradient (5 to 40 % ethyl acetate). The fractions were monitored by TLC, and the fractions containing the main product were combined and dried by rotary evaporation. The product was confirmed by 1H-NMR and mass spectral analysis. The yield was 2.9 g or 70% for the two steps. 1H-NMR($CDCl_3$, 200 MHz), δ(ppm) 7.0-8.6 (m, 5H, Ar-H and -NH), 5.809 (s, 1H, one of $=CH_2$), 5.361 (s, 1H, one of $=CH_2$), 3.603 (m, 2H, $-CH_2-NR$), 2.960 (t, 2H,- $S-CH_2-$), 2.002 (d, 3H, $-CH_3$, the split of this peak is due to the tautomerization of the adjacent double bond). **MS (FAB),** *m/e* 255 (M+1).

Synthesis of pyridine disulfide modified HPMA co-polymer

HPMA, PDTEMA and AIBA were dissolved in acetone (12.5 wt % monomers, 0.6% wt % AIBN). Insoluble impurities were removed by filtration or centrifugation. The solution was bubbled with N_2 for 5 minutes to remove the

dissolved oxygen and the reaction vessel was closed tightly. The reaction was placed in a 50-55° C water bath for 24 hours. The white precipitate formed was recovered by filtration, and was dried in a vacuum desiccator. The crude product was dissolved in a small volume of MeOH (approx. 1 g polymer in 4 mL MeOH), then precipitated by slowly adding excess acetone. The MeOH/acetone precipitation was performed twice. After the last precipitation, the polymer was dried in a desiccator until the weight was constant and then stored at -20° C. The mole percentage of pyridine ring in each polymer was quantified by the UV absorption at 281 nm using an extinction coefficient of 4900 in pH 7.0 aqueous solution.

Synthesis of amino and pyridine disulfide modified HPMA copolymer

HPMA, PDTEMA, APMA and AIBN were dissolved in methanol (12.5 wt % monomers, 0.6% wt % AIBN). The solution was bubbled with N_2 for 5 minutes, and the reaction vessel was sealed. The tube was placed in a 50-55° C water bath for 24 hours. Insoluble impurities were removed by filtration or centrifugation. Excess acetone was added to the clear solution to precipitate the co-polymer product. The white product was purified by re-precipitation and stored as described above.

Hydrolysis of pyridyl disulfide modified HPMA co-polymer

The kinetics of hydrolysis were measured using a dual beam spectrophotometer. Sample and blank cuvettes contained 0.1 M buffer A or B, depending on the desired pH. To the sample cuvette polymer A was added to a final concentration of 0.23 mg/mL. After addition, the absorbence at 320 nm (pH equal to or greater than 11) or 341 nm (pH equal to or less than 10) was measured at 2 minute intervals for 14 hours at room temperature.

Kinetics of the reaction between polymer A and sulfhydryl peptide

A sample and blank cuvette were filled with 0.1 M buffer A, and polymer A was added to the sample cuvette to a final concentration of 0.23 mg/mL. pAntp-SH (in 0.1% TFA) was added to both cuvettes to a final concentration of 0.50 mM and the absorbence was measured at 341 nm every 0.5 minutes for 50 minutes. The free sulfhydryl in pAntp-SH was quantified using Ellman's reagent .

Kinetics of the conjugation between the HPMA co-polymer and oligonucleotides

Ten nmoles of disulfide modified oligonucleotide (RSS-OSH) were treated with 0.2 M DTT in 100 μL of 0.1 M pH 9.0 buffer B for 2 hours. Excess DTT was removed by ethyl acetate extraction (3 x 100 μL). The oligonucleotide was precipitated by adjusting the solution to 0.3 M NaOAc and adding 2.6 volumes of ethanol. The mixture was placed at -20° C for 1 hour and centrifuged for 25 minute at 600xg. The oligonucleotide pellet was dissolved in 80 μL de-ionized water, and 10 μL of 1.0 M buffer and 20 nmoles (in pyridine ring) of co-polymer A were added. The reaction was incubated at room temperature. Ten μL of the reaction mixture was removed at different times, stopped and immediately loaded into a running 20% PAGE. The gel was illuminated with UV light for fluorescence visualization. To confirm that oligonucleotide was covalently conjugated to HPMA polymer in the reaction product, 10 μL of the 30 minute reaction mixtures were treated with 0.1 M DTT for 30 minute at room temperature and loaded on the gel together with the 60 minute reaction sample.

Preparation of oligonucleotide-polymer conjugate

100 nmoles of disulfide modified oligonucleotide (RSS-OSH) were treated with 0.2 M DTT in 300 μL of 0.1 M pH 9.0 buffer B for 2 hours. Excess DTT was removed by ethyl acetate extraction (3 x 300 μL), and the oligonucleotide was precipitated by adjusting the solution to 0.3 M NaOAc and adding 2.6 volumes of ethanol. The mixture was placed at-20° C for 1 hour and centrifuged for 25 minutes at 600xg. The oligonucleotide pellet was dissolved in 100 μL of de-ionized water, and 200 nmole (in pyridine ring) of active HPMA co-polymer and 30 μL of pH 9.0 buffer B were added. The mixture was brought to a final volume of 300 μL with de-ionized water and incubated at room temperature for 2 hours. The pH of the reaction mixture was adjusted to about 7 using 1.0 M HCl to inhibit disulfide exchange. L-Cysteine was added to the reaction mixture to a final concentration of 1.0 mM and incubated at room temperature for 10 minutes to destroy the excess pyridyl disulfide bonds in the co-polymer. At pH 7 L-cysteine reacts rapidly and completely with all unreacted pyridyl disulfide linkages (data not shown). The product was purified by passing the reaction mixture through a short G-50 column. The reactions were

characterized using 20% PAGE. Eighty percent of the oligonucleotide was conjugated to the HPMA polymer as indicated by its inability to enter the gel. The conjugation product was concentrated to a small volume using a Microcon-30 filter.

Preparation of oligonucleotide-peptide-polymer conjugate

The oligonucleotide-peptide-polymer conjugate was prepared as for the oligonucleotide-polymer conjugate, except that 200 nmoles (in pyridine ring) of HPMA co-polymer was first reacted for 10 minutes at room temperature with 20 nmoles of pAntp-SH peptide in 0.1 M pH 9.0 buffer B. Subsequently, the oligonucleotide was added. The product was worked up as described above.

Results and Discussion

Synthesis of pyridyl disulfide modified HPMA copolymers

N-[2-(2-pyridyldithio)ethyl methacrylamide (PDTEMA) was synthesized through a two step, one-pot reaction, as illustrated in Figure 1. Cysteamine reacted completely with DPDS in an ethanol/water mixture at room temperature in less than one hour. Although 2-(2-pyridyldithio)ethyl amine (PDTEA) has reportedly been crystallized from benzene, the dried crude product was used directly in the second step. Subsequently, PDTEMA was purified by silica gel chromatography in an overall yield of greater than 70%. The structure was confirmed by UV, 1H-NMR and MS.

PDTEMA co-monomer was incorporated into HPMA polymer by precipitation radical polymerization with HPMA monomer in acetone at 50-55° C . Co-polymers varying in the percentage of active pyridyl disulfide groups were synthesized. The mole percent of pyridine ring in each polymer was quantified by the UV absorption at 281 nm using a molar extinction coefficient of 4900 at pH 7.0 in aqueous solution. The percent conversion of PDTEMA in all the syntheses was similar (Table 1), indicating the pyridyl group of PDTEMA does not significantly affect its incorporation into the HPMA co-polymer. This also suggests that there was no significant degradation of the active pyridyl disulfide moiety during the polymerization reactions. Since the structure of the co-monomers are very similar, in terms of the active double bond, the pyridyl disulfide moieties are expected to be

distributed homogeneously in the polymer molecules. This is confirmed by the overlapping UV detection and fraction index detection curves in the chromatogram of the gel permeation chromatography of th polymer (Figure 2). This indicates that the percentage of pyridyl groups is similar throughout the full size range of molecules within the same polymer batch.

Amine/pyridyl disulfide containing polymers could also be synthesized by the inclusion of APMA in the polymerization reaction (Table 2). Since APMA is supplied as a hydrochloride salt, and is insoluble in acetone, APMA containing reactions were performed in methanol solutions. The ability to incorporate both groups into the polymer allows conjugation of different moieties to the polymer using different chemistries.

Aqueous stability and reactivity of pyridyl disulfide side chains in the co-polymer

The UV absorbence spectra of the 2-pyridyl disulfide containing HPMA polymer and the PDTEMA monomer are similar to that for 2,2'-dipyridyl disulfide (DPDS). DPDS has a maximum absorption at 284 nm, which is substantially different from the UV spectrum of 2-thiopyridone under the same conditions (Figure 3). 2-Thiopyridone has a maximum absorption at 341 nm and this is relatively constant below a pH of 8. Between pH 9 and 11 the spectrum changes drastically (Figure 3). This is likely the result of the ionization of the nitrogen proton which has a pKa of approximately 10, and the presence of the 2-pyridine-thiolate ion at pH higher than 10. Since the absorbence at 320 nm is relatively high and constant for 2-thiopyridone, and at a minimum for the pyridyl disulfide group, the absorbence at 320 nm was used to monitor release of 2-thiopyridone from the polymer at pH greater than 10. For pH equal to or less than 10 the absorption maximum at 341 nm was used to monitor the release of 2-thiopyridone from the co-polymer.

The pyridyl disulfide bond is very stable in de-ionized water (pH = 6.5), there is no detectable release of 2-thiopyridone after storage for 2 months at -20° C in a 20 mM (in pyridine ring) polymer stock solution. The kinetics of hydrolysis/aminolysis were analyzed at room temperature using 0.1 M glycine/NaOH buffers over a wide range of pH values (Figure 4). The stability of the pyridine disulfide bond in the co-polymer decreases with increasing pH in aqueous solution. There was no detectable

degradation of the active disulfide bond within 14 hours in 0.1 M pH 8.0 citric acid/NaOH buffer. Incubation for the same time at pH 9.0 and 10.0, in 0.1 M glycine/NaOH buffered solution, released approximately 11% and 17% of the active pyridine group, respectively. Above a pH of 10 the degradation increased significantly with a half life of hydrolysis and/or aminolysis of approximately 25 minutes in pH 13 glycine buffer. These results indicate that pyridyl disulfide modified HPMA copolymer can be safely stored and/or further derivatized in aqueous solutions of pH equal to or less than 8. If necessary, the excess active pyridyl disulfide bond can be quickly destroyed by increasing the pH to 13 or higher.

The reactivity of the pyridyl disulfide activated HPMA co-polymers with thiol groups was first examined using the model compounds L-cysteine and 2-mecaptoethanol. Similar to DPDS, SPDP and other small molecule active pyridyl disulfide compounds, the active pyridyl group in HPMA copolymer can be replaced by L-cysteine or 2-mecaptoethanol in aqueous solutions in a wide range of pH values (data not shown). At neutral pH the reaction is rapid and complete, and disulfide exchange is suppressed.

Characterization of the conjugation reaction of HPMA co-polymer with peptide and/or oligonucleotide

To examine the reactivity of the activated polymer with larger bioactive molecules a moderate sized peptide (pAntp-SH, Fw = 2407) was first examined (Figure 5). pAntp-SH is 16 amino acids long and possesses a cysteine residue at its carboxyl terminus. pAntp-SH is derived from the homeodomain of Antennapedia. pAntp was chosen since it has been shown to translocate across biological membranes in an energy independent and non-endocytotic manner. When conjugated to a bioactive peptide or antisense oligonucleotide, pAntp has been demonstrated to enhance uptake and activity of these molecules. Similar to cysteine and 2-mecaptoethanol, pAntp-SH can readily react with pyridyl disulfide HPMA co-polymer in aqueous solutions. As seen in Figure 6, the reaction goes to completion within minutes in a mildly basic solution. The rate of reaction decreases with decreasing pH, but even in acidic solution the rate of reaction is relatively high. The half life of reaction is less than 50 minutes at pH 3.

It is believed that the protonation of the pyridine ring nitrogen makes the pyridyl group a good leaving group in pyridyl disulfide mediated thiol-disulfide exchange reactions. This is in contrast to thiol-disulfide exchange reactions between aliphatic thiols and aliphatic disulfides, where the thiolate ion, rather than the thiol, is the reactive species in the nucleophilic displacement reaction. Since the pKa of most aliphatic thiols is between 7 and 9, little or no reaction occurs in acidic solutions, owing to the low thiolate ion concentration.

An oligonucleotide targeted to the 3' splice junction of the second intron of the HIV tat gene was selected for examination of the conjugation of an antisense oligonucleotide to the polymer (Figure 5, RSS-OSH, Fw = 7130). This oligonucleotide was chosen since in a examination of 20 different targets on HIV, this target was one of the more effective ones at inhibiting viral replication. To increase the resistance of the oligonucleotide to nucleolytic degradation, all phosphodiester linkages, were substituted with phosphorothioates, and a 3'-3' inverted cap was added to the 3'-end during chemical synthesis. A fluorescein group was added to the 5'-end, followed by a linker and a disulfide group.

The disulfide group on the oligonucleotide was reduced to produce a free thiol using DTT. Subsequently, the oligonucleotide was allowed to react with the pyridyl disulfide modified copolymer. Since the oligonucleotide has a strong UV absorbence between 200 and 300 nm, UV absorbence could not be used to monitor the course of the reaction. Instead gel electrophoresis was used. We examined the kinetics of conjugation of RSS-OSH with the 2-pyridyl disulfide modified polymer at pH 7.0 to 10 (Figure 7). The reaction was allowed to proceed for up to 20 hours, and aliquots were removed at various times, combined with an equal volume of gel loading dye and immediately loaded onto a denaturing 20% polyacrylamide gel. Since the polymer is a large neutral molecule, the polymer-oligonucleotide conjugate barely migrates into the gel, while the mobility of the free oligonucleotide is very fast. The different apparent mobility's of the free oligonucleotide, at various times, is due to the different times in which each sample was loaded on the gel. As seen in Figure 7, the conjugation was inefficient until the pH of the solution reached 8.0. When the pH was 7.0, less than half of the oligonucleotide coupled to the polymer after incubation for 20 hours (Figure 7). However, the half life of the conjugation was reduced to

about 10 and 5 minutes, respectively, when the reaction was performed at pH 8.0 or 9.0. The reaction was 50% complete in minutes when performed at pH 10.0.

Preparative conjugation of HPMA co-polymer with peptide and/or oligonucleotide

All preparative conjugation reactions were conducted in pH 9.0 buffer in the presence of excess pyridyl disulfide groups of the HPMA copolymer. For peptide/oligonucleotide conjugates, the peptide was conjugated first followed by the oligonucleotide. After completion of the conjugation, the pH of the reaction mixture was adjusted to about 7, and excess pyridyl disulfide groups were inactivated by addition of L-cysteine to a final concentration of 1.0 mM. The conjugation products could be easily separated from the free oligonucleotide and peptide as a result of the increased size of the polymer conjugate. The conjugate was characterized by PAGE and conjugation was confirmed by release of the oligonucleotide after treatment with DTT (data not shown). The UV spectrum of the oligonucleotide-HPMA polymer is exactly the same as the free oligonucleotide, while there was a small change in the shape of the spectrum for the pAntp peptide/oligonucleotide conjugate (figure 8). The ratio of the optical densities at 260 and 280 nm was significantly higher for the oligonucleotide-peptide-HPMA conjugate (1.77) than for the oligonucleotide-HPMA conjugate (1.51), indicating the presence of peptide in the former terplex conjugate. The structure of the oligonucleotide-peptide-HPMA terplex conjugate was further confirmed by DTT reduction. An orange precipitate formed after the addition of 0.2 M DTT in pH 9.0 buffer to the terplex conjugate. The precipitate is likely a polyelectrolyte complex between oligonucleotide and peptide resulting from electrostatic interaction of the 21 negative charges on the oligonucleotide and the 7 to 10 positive charges on the peptide (4 from lysine's, pK_a=10.69; and 3 to 6 from arginine's, pKa_1=12.48 and pKa_2=8.99). This was confirmed by the immediate formation of an orange precipitate on combining equal amounts of free oligonucleotide and peptide in pH 9.0 buffer.

Cell uptake of the HPMA-oligonucleotide conjugate

The uptake, by mammalian cells, of the oligonucleotide-HPMA conjugate was examined using the HeLa S3 cell line. Cells were pre-cultured in DMEM

medium containing 10% fetal bovine serum on sterilized cover slips. The cells were plated to give approximately 10% coverage in 2 to 3 days. The media was removed and the cells washed with fresh media. The media was replaced and the free oligonucleotide or the oligonucleotide polymer conjugate was added to a final oligonucleotide concentration of 5 μM in oligonucleotide. After incubation for 5 hours, the cells were rinsed with PBS three times, fixed using acetone, and analyzed by confocal microscopy.

Uptake of the polymer-oligonucleotide conjugate is mildly enhanced relative to the free oligonucleotide. As shown in Figure 9, most of the fluorescence of the oligonucleotide is present in endosomes as is typically observed for endocytotic uptake of antisense oligonucleotides or HPMA polymers. To confirm an endocytotic mode of uptake the experiments were repeated except that incubation in the presence of oligonucleotide or oligonucleotide-polymer conjugate was performed at 4°C. In this case, little or no internalized fluorescence was observed, indicative of a lack of significant uptake.

Since the oligonucleotide has a 3' inverted cap and is composed of phosphorothioate linkages, it is expected to be stable toward nuclease degradation. In fact, for the free oligonucleotide, it remained 90% intact after 24 hours at 37 °C in 50% calf serum (data not shown). Stability should be further enhanced for the conjugated oligonucleotide as its 5' end is blocked by coupling to the polymer. It is therefore likely that the internalized oligonucleotide remains primarily intact.

These results provide evidence to support the value of the copolymer of the invention for delivery of antisense oligonucleotides. The effectiveness of this delivery system can be further enhanced in a variety of ways. First, in the uptake studies, the polymer was only loaded at a low level, approximately one oligonucleotide per molecule. Since the polymer contains 6 to 20 active thiols, depending on the mole % of PDTEMA in the polymer feed, higher loading will likely lead to further enhancement of uptake. Second, release of the oligonucleotide from endosomal compartments would certainly improve antisense efficacy. The coupling of a lysogenic agent, such as a fusogenic peptide, to the polymer could aid in this regard. A fusogenic peptide could be coupled to the polymer using the activated sulfhydryl. Alternatively, the use of APMA/PDTEMA co-polymer could allow different

chemistries to be used for the oligonucleotide and lysogenic agent. Finally, targeted delivery would also enhance efficacy. This could be accomplished by coupling a targeting moiety through the activated thiol, or alternatively, in the case of an amino-containing (e.g. APMA/PDTEMA) co-polymer, through the amino groups. The use of both a targeting moiety and a lysogenic agent would likely further enhance efficacy.

Activity could likely also be enhanced by internalization of the conjugate by non-endocytotic means. This would prevent exposure of the oligonucleotide to the degradative enzymes of the lysosome. Additionally, direct cytoplasmic uptake would likely lead to rapid nuclear accumulation of the oligonucleotide. It has been shown that a short peptide (pAntp) from the Antennapedia homeodomain protein can translocate across biological membranes in an energy independent, non-endocytotic manner. Uptake of peptides and oligonucleotides has been reported to be increased greater than 100 fold when coupled to pAntp. This has proven ineffective for oligonucleotides. When an anti-HIV oligonucleotide is coupled directly to pAntp, a conjugate was produced that was only soluble at pH 13 and greater. The is believed to be due to the formation of an intramolecular polyelectrolyte complex. This could result from interaction of the approximately ten positive charges on pAntp and the 20 negative charges on the oligonucleotide at neutral pH, as discussed above. To solve the solubility problem we coupled pAntp to the copolymer of the invention and subsequently conjugated the oligonucleotide. While this solved the solubility problem, we saw no alteration in the amount of uptake or the intracellular distribution of the oligonucleotide-pAntp-polymer conjugate. Again, this is likely the result of electrostatic association of peptide and oligonucleotide. This could prevent electrostatic interaction of the peptide with the cell membrane, which may be essential to its correct functioning. Additionally, this would likely prevent proper folding of the peptide into a structure necessary for translocation. It therefore seems unlikely that pAntp can be used successfully for translocation of negatively charged oligonucleotides, unless a delivery system can be developed that allows the peptide and oligonucleotide to be physically separated.

2-Pyridyl disulfide homogeneously modified HPMA co-polymer was synthesized by co-polymerization of PDTEMA and HPMA. This is believed to be the easiest and probably the only way, to date, to synthesize homogeneous thiol or active

thiol polymers. Most of the available thiol-polymers were synthesized by derivatizing pendant hydroxyl or other functional groups on various hydrophilic polymers.

The pyridyl disulfide activated co-polymer is stable in aqueous solutions of pH lower than 8.0 and can readily react with thiol containing molecules in aqueous solutions of a wide range of pH. Consequently, it provides a very mild and efficient way to conjugate water soluble molecules to HPMA polymer. Coupling of a drug through a disulfide linkages provides other advantages as well. Disulfide linkages have been shown to be stable in the blood stream so the drug will remain with its carrier until it reaches its site of action. Subsequently, the drug can be released by disulfide exchange with intracellular thiols such as glutathione, or alternatively by the action of redox enzymes. Since drug release is not dependent on any enzymatic activity, the route of uptake, endocytotic or non-endocytotic, should have little effect on release. This can be very important since enzyme dependent release likely requires endocytotic uptake and lysosomal targeting. This places the drug, an oligonucleotide of the present invention, in contact with degradative enzymes. The use of a fusogenic peptide on the drug carrier could limit lysosomal degradation. However for other delivery systems that require enzymatic release of the drug, inclusion of a fusogenic peptide could also prevent drug release.

The co-polymers of the invention have many attributes that make it a valuable alternative for delivery of water soluble drugs such as antisense oligonucleotides. The utility of these polymers is further enhanced by the ability to incorporate pendant amino groups as well. It is also possible that p-nitrophenol or N-succinimidyl active ester groups could also be introduced during polymerization. This allows great flexibility in the design of an effective drug delivery system. The drug, a targeting moiety, a fusogenic peptide and any other desirable functionality can be coupled using a variety of chemistries. For the PDTEMA/HPMA co-polymer alone, there are different chemistries that can be employed. Aside from the obvious coupling of thiol-containing molecules through the activated pyridyl disulfide, one can reduce the pyridyl disulfide linkage and couple directly through the free sulfhydryl group. In this way non-degradable linkages can be produced by reaction with maleimido or iodoacetal derivatized ligands.

While this invention has been described with reference to certain specific embodiments and examples, it will be recognized by those skilled in the art that many variations are possible without departing from the scope and spirit of this invention, and that the invention, as described by the claims, is intended to cover all changes and modifications of the invention which do not depart from the spirit of the invention.

Table 1

Analysis of Pyridyl Disulfied Modified HPMA Copolymers

	Mole% PDTEMA					
Copolymer	Feed	Polymer	Conversion %	M_r (number)	M_r (weight)	polydispersity
A	11.8	8.3	82	19 504	38 001	1.95
B	5.4	3.7	81	27 672	82 533	2.98
C	2.6	1.6	73	28 987	80 020	2.76
D	1.8	1.2	79	28 851	75 373	2.62

Table 2

Analysis of HPMA Copolymer Containing Pyridyl Disulfide and Amino Groups

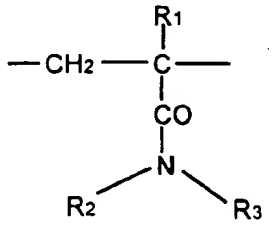
	Mole %								
	Feed		Polymer		Conversion %				
Polymer	NH_2	Py	NH_3	Py	NH_2	Py	M_r (number)	M_r (weight)	polydispersity
E	1.01	1.41	1.02	1.13	100	80.1	23 640	36 980	1.56
F	0.95	7.3	0.90	5.5	95	75.3	15 640	23 840	1.52
G	5.8	1.2	5.0	0.87	86.2	72.5	8 430	10 540	1.25

CLAIMS

What is claimed is:

1. A copolymer comprising,

substituted acrylamide monomeric units which are the same or different and of the formula;



where

R_1 is H, methyl or ethyl,

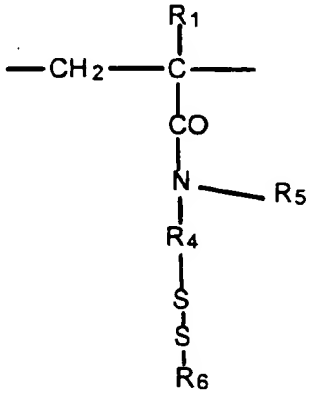
R_2 and R_3 are the same or different and are

alkyl having one to eight carbon atoms or alkoxyalkyl having one to twelve carbon atoms and one to three -OH groups, or

hydroxyalkoxyalkyl of two to twelve carbon atoms and one to three hydroxy groups, or

aminoalkyl having two to twelve carbon atoms and one to three amino groups, and

pyridyldithio-acrylamide monomeric units which are the same and different and of the formula;



where R_4 is

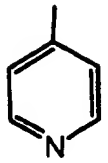
an alkyl chain having one to eight carbon atoms or alkoxyalkyl having one to twelve

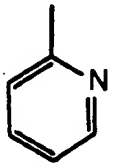
carbon atoms and zero to three -OH groups, or

an hydroxyalkoxyalkyl chain of two to twelve carbon atoms and zero to three hydroxy groups, and

R_5 is H, methyl, or ethyl,

R_6 is





or

the mole percent of the substituted acrylamide monomeric units in the copolymer being between 70 and 99 the remaining monomeric units being pyridyldithio-acrylamide monomeric units.

2. The copolymer of Claim 1 wherein R_2 is an alkyl or alkoxyalkyl, and R_3 is H, methyl or ethyl.

3. The copolymer of Claim 1 wherein R_4 is an alkyl or alkoxy, and R_5 is H.

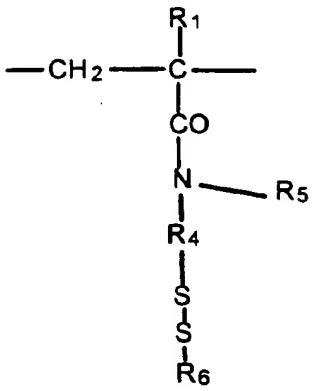
4. The copolymer of Claim 1 wherein R_1 is methyl, R_2 is hydroxypropyl, and R3 is H

5. The copolymer of Claim 1 wherein R_1 is methyl, R_4 is ethyl, and R_5 is hydrogen.

6. The copolymer of Claim 1 wherein for all or at least a portion of the monomeric units R_3 is amino-alkyl.

7. The copolymer of Claim 1 wherein for all or at least a portion of the monomeric units R_3 is aminopropyl.

8. A copolymer comprising acrylamide monomeric units and from 1 to 30 mole percent of the monomeric unit,



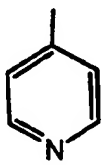
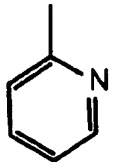
where R_4 is

an alkyl chain having one to eight carbon atoms or alkoxyalkyl having one to twelve

carbon atoms and one to three -OH groups, or

an hydroxyalkoxyalkyl chain of two to twelve carbon atoms and one to three hydroxy groups,

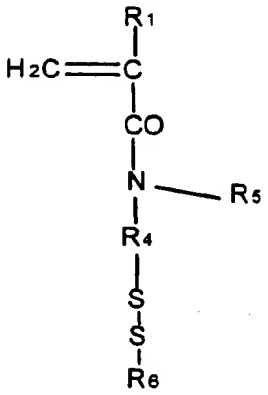
R_5 is H, methyl, or ethyl,

and R_6 is  or 

9. The copolymer of Claim 8 wherein R_4 is an alkyl or alkoxy, and R_5 is H.

10. The copolymer of Claim 8 wherein R_1 is methyl, R_4 is ethyl, and R_5 is hydrogen.

11. A composition

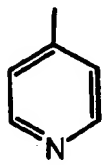
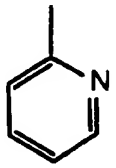


where R_4 is

an alkyl chain having zero to eight carbon atoms or alkoxyalkyl having one to twelve carbon atoms and zero to three -OH groups, or

an hydroxyalkoxyalkyl chain of two to twelve carbon atoms and one to three hydroxy groups, and

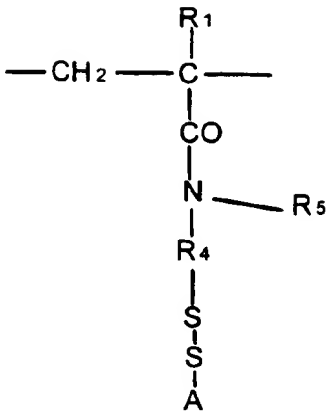
R_5 is H, methyl, or ethyl,

R_6 is  or 

12. The composition of Claim 11 wherein R_4 is an alkyl or alkoxy, and R_5 is H.

13. The composition of Claim 11 wherein R_1 is methyl, R_4 is ethyl, and R_5 is hydrogen.

14. A conjugate of an agent having an active sulfhydryl group and a copolymer the copolymer comprising monomeric units of the formula bonded to a derivative of the agent A through a disulfide bond,



where R_4 is

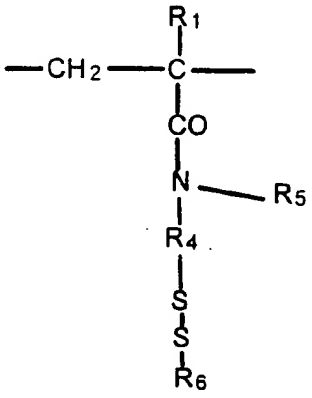
an alkyl chain having one to eight carbon atoms or alkoxyalkyl having one to twelve carbon atoms and zero to three -OH groups, or

an hydroxyalkoxyalkyl chain of two to twelve carbon atoms and zero to three hydroxy groups, and

R5 is H, methyl, or ethyl.

15. A process for producing a conjugate comprising;

forming a copolymer of a acrylamide monomeric units and from 1 to 30 mole percent of the monomeric unit,



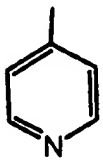
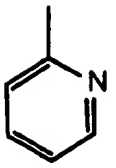
where R_4 is

an alkyl chain having one to eight carbon atoms or alkoxyalkyl having one to twelve

carbon atoms and zero to three -OH groups, or

an hydroxyalkoxyalkyl chain of two to twelve carbon atoms and zero to three hydroxy groups, and

R_5 is H, methyl, or ethyl,

R_6 is  or  ,

contacting the copolymer with an agent in an aqueous environment, the agent containing at least one free sulfhydryl group to form a conjugate of the copolymer through a disulfide bond.

16. The process of Claim 15 wherein R_4 is an alkyl or alkoxy, and R_5 is H.

17. The process of Claim 15 wherein R_1 is methyl, R_4 is ethyl, and R_5 is hydrogen.

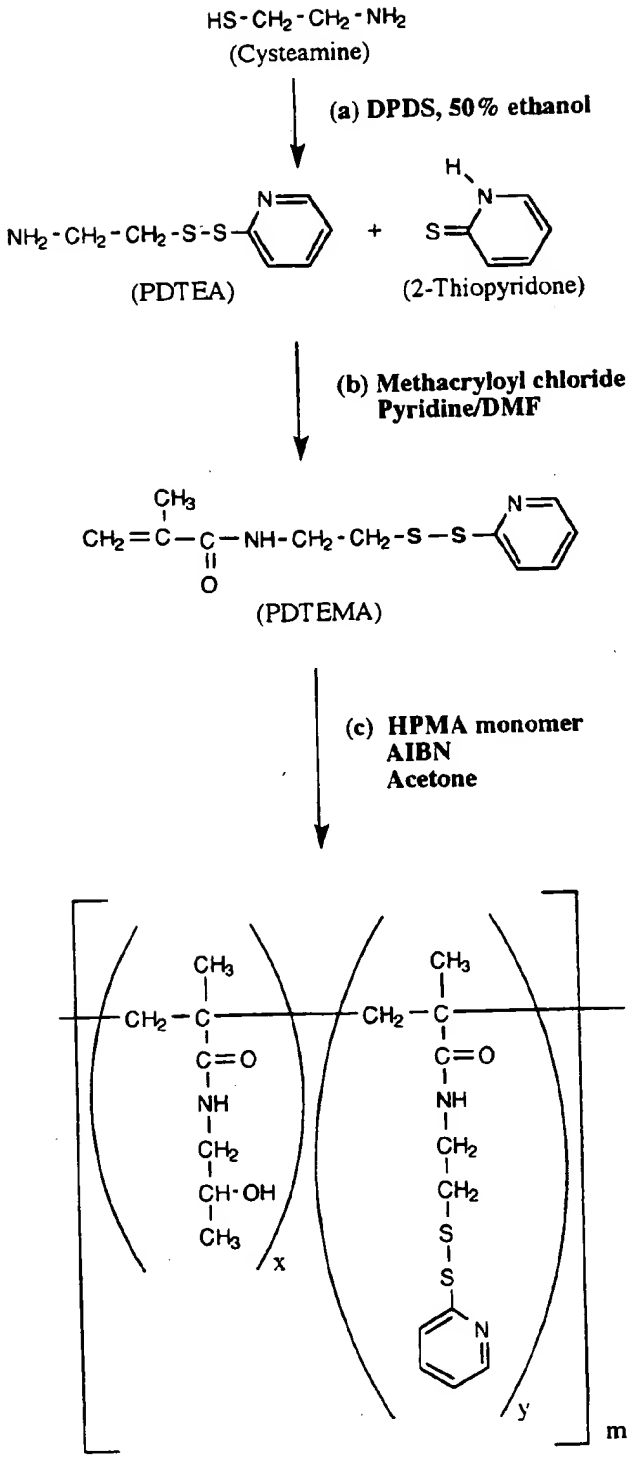

HS-CH2-CH2-NH2
(Cysteamine)
(a) DPDS, 50% ethanol
NH2-CH2-CH2-S-S
(PDTEA)
(2-Thiopyridone)
(b) Methacryloyl chloride
Pyridine/DMF
(PDTEMA)
(c) HPMA monomer
AIBN
Acetone
x
y
m

Fig. 1

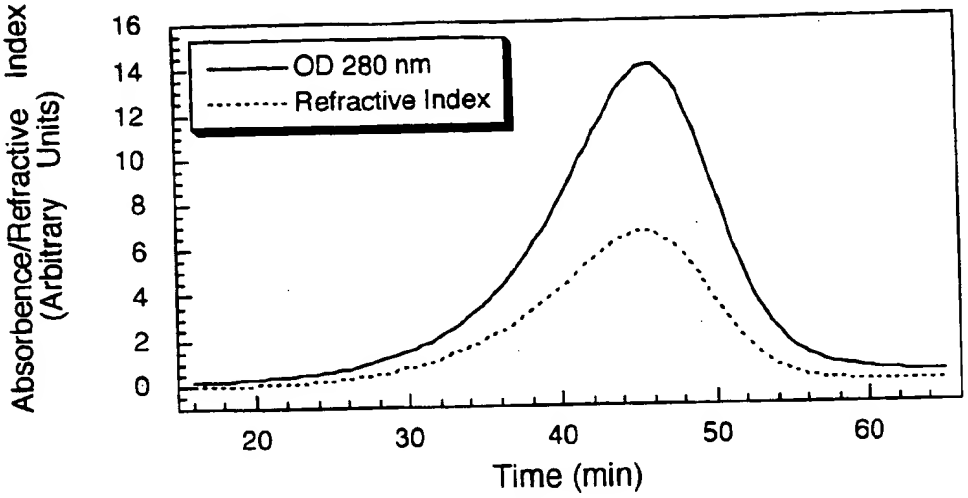

Absorbence/Refractive Index (Arbitrary Units)
16
14
12
10
8
6
4
2
0
OD 280 nm
Refractive Index
20
30
40
50
60
Time (min)

Fig. 2

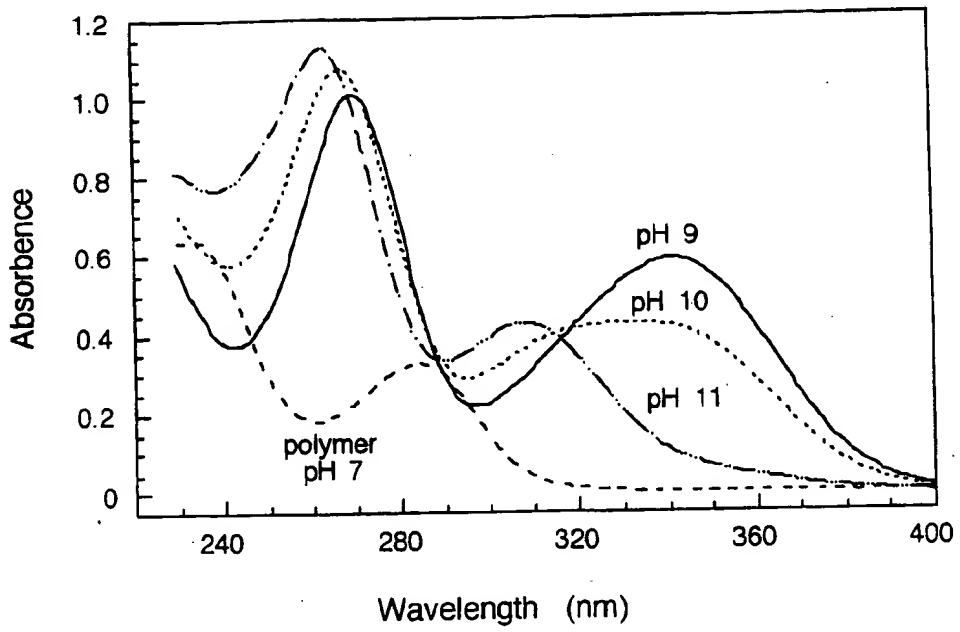

1.2
1.0
0.8
0.6
0.4
0.2
0
Absorbence
240
280
320
360
400
Wavelength (nm)
pH 9
pH 10
pH 11
polymer
pH 7

Fig. 3

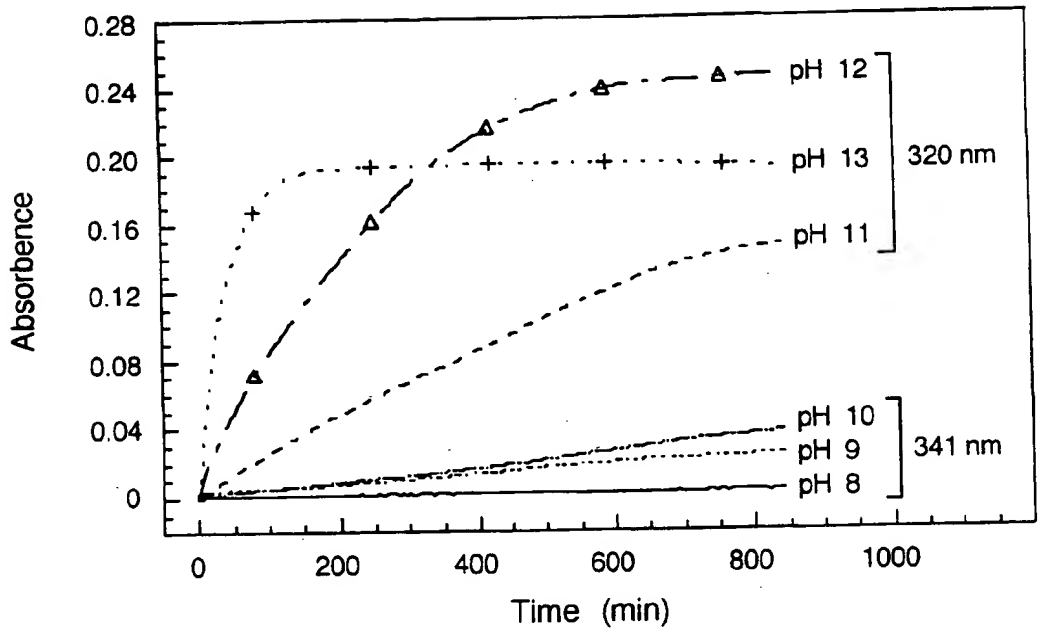
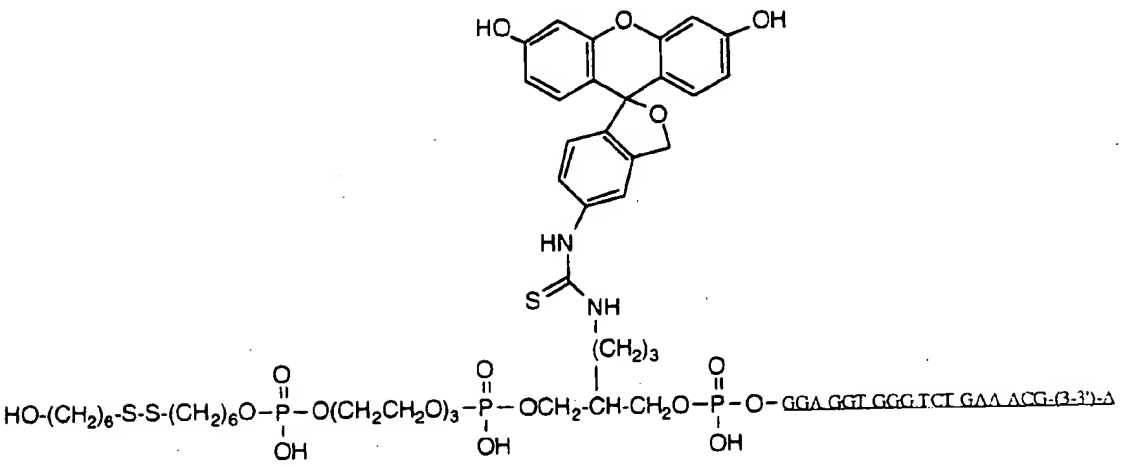

0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
Absorbence
0
200
400
600
800
1000
Time (min)
pH 12
pH 13
pH 11
320 nm
pH 10
pH 9
pH 8
341 nm

Fig. 4

Cys-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys

pAntp-SH



RSS-OSH

Fig. 5

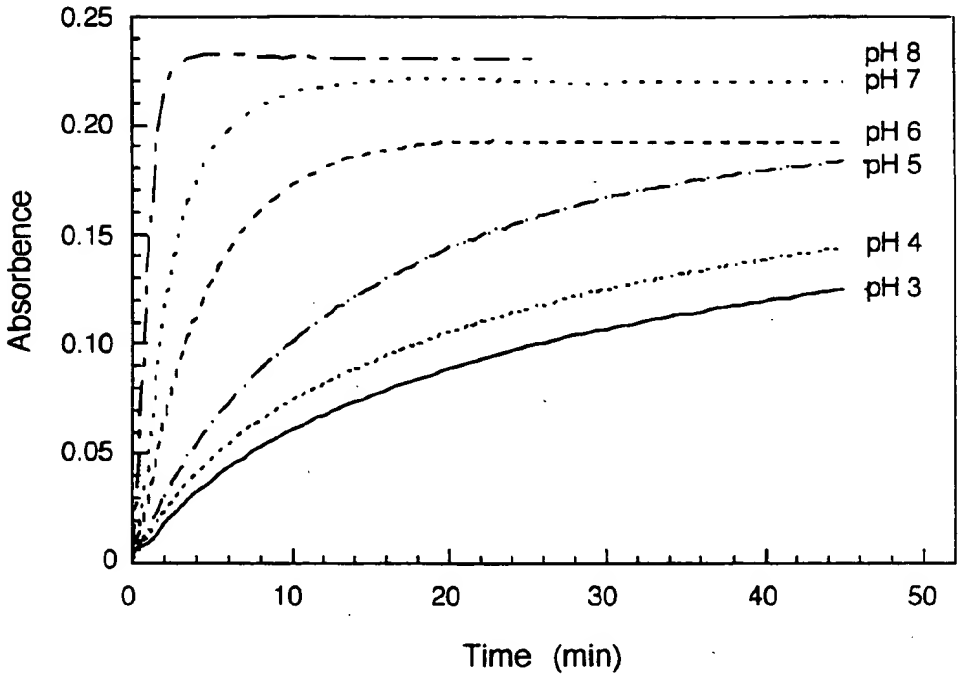
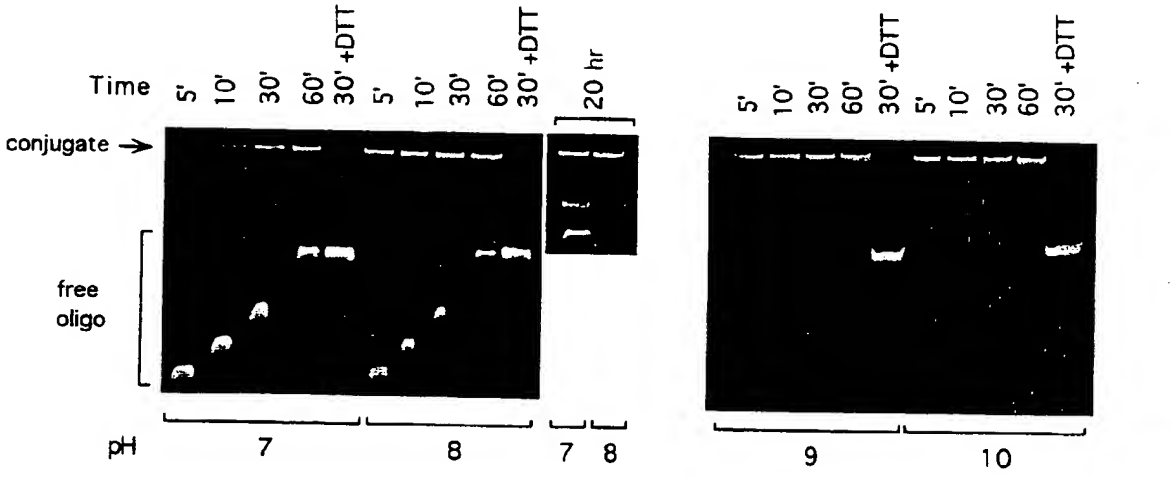

0.25
0.20
0.15
0.10
0.05
0
Absorbence
0
10
20
30
40
50
Time (min)
pH 8
pH 7
pH 6
pH 5
pH 4
pH 3

Fig. 6

Figure 7


Time
5'
10'
30'
60'
30' +DTT
5'
10'
30'
60'
30' +DTT
20 hr
conjugate
free
oligo
pH
7
8
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10'
30'
60'
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5'
10'
30'
60'
30' +DTT
9
10

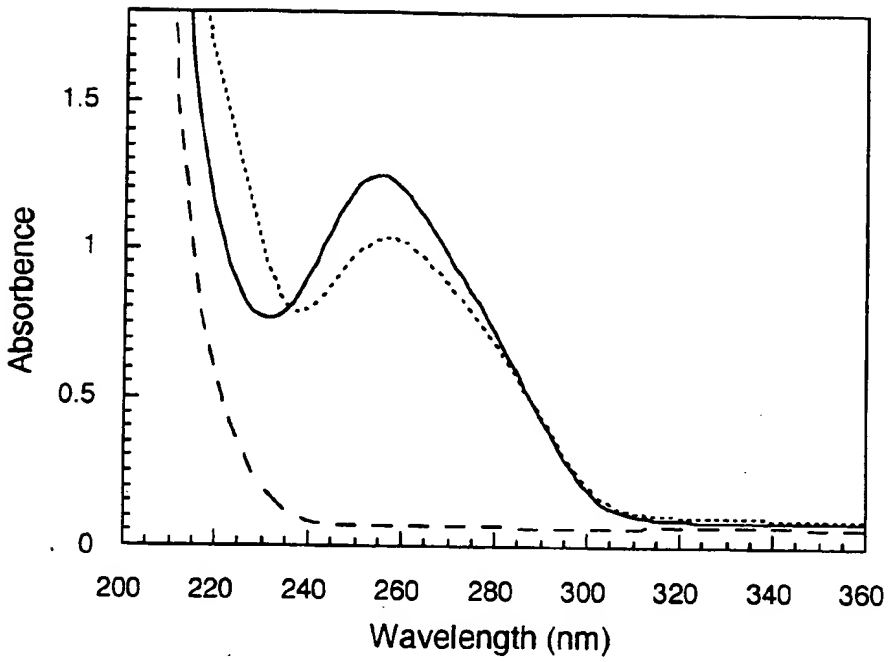
Absorbence
1.5
1
0.5
0
200
220
240
260
280
300
320
340
360
Wavelength (nm)

Fig. 8

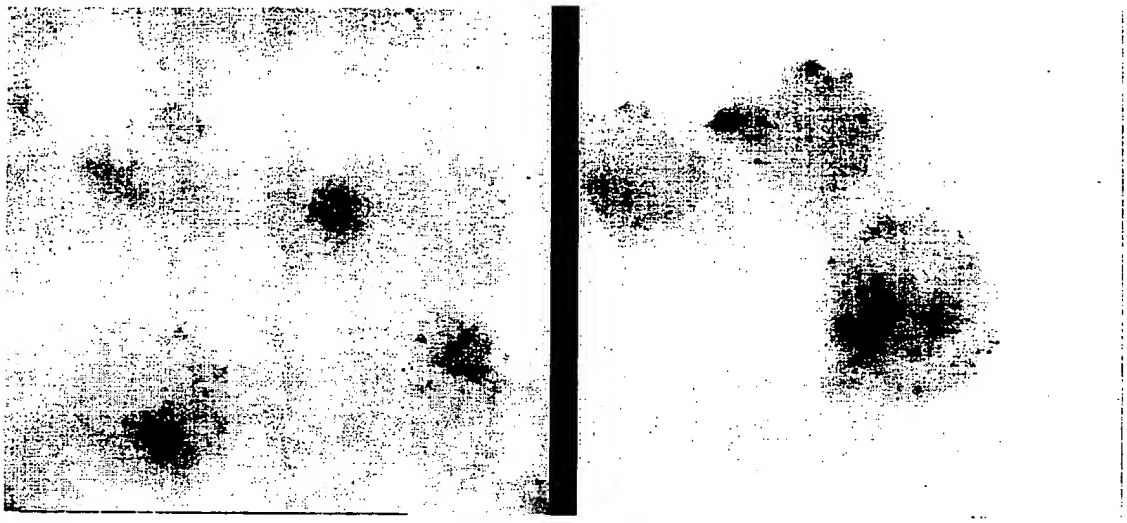

Fig. 9

INTERNATIONAL SEARCH REPORT

.rnational application No.
PCT/US00/29849

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07D 211/72; C08F 126/06

US CL :526/265; 546/294

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 526/265; 546/294

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,863,789 A (KOMATSU et al) 26 January 1999.	1-17
A	US 5,118,681 A (AMICK et al) 02 June 1992.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
16 JANUARY 2001	25 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer BERNARD LIPMAN
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